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TITLE: Cell Adhesion-Linked Protein Tyrosine Phosphatases and
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13. ABSTRACT (Maximum 200 Words) <p>21 "classical" protein tyrosine phosphatases (PTPs) were identified in human mammary epithelial cell (MEC) lines. Degenerate RT-PCR followed by restriction fragment differential display (RFDD) and specific RT-PCR were used to assess expression in the continuous HMT-3522 cell series that includes both non-malignant S1 and tumorigenic T4-2 cells in monolayer and during normal and dysregulated morphogenesis in EHS-ECM (Matrigel). PTP expression was generally higher in tumorigenic T4-2 cells and unchanged by disorganized growth in Matrigel. In contrast, coordination of expression was suggested by the transient upregulation (relative to monolayer cultures) of a number of PTPs during acinar morphogenesis of non-malignant S1. The kinetics of downregulation for some suggested that growth arrest may be the main regulatory input. Others however, downregulate with more rapid kinetics before significant growth arrest suggesting different regulatory inputs. Feedback from cell-cell adherens junctions (AJ) may be one such input as ectopic expression of a dominant negative E-cadherin construct that blocks AJ formation delayed but did not prevent downregulation of selected PTPs. Modest upregulation of actin cytoskeleton regulating PTPs in response to decreases in substrate compliance occur when normal MCF10A were plated on (~1 order of magnitude) softer (tissue-like) Matrigel coupled polyacrylamide gels suggesting that these PTPs may be responding to, or mediating corresponding actin cytoskeletal reorganization. PTPN12 is a cytoplasmic PTP highly expressed in MECs that localizes transiently to actin polymerizing zones including lamellopodial leading edges and the metaphase mitotic spindle and plasma membrane. Stable downregulation of PTPN12 by retroviral mediated shRNAi resulted in derangements of the actin cytoskeleton in MCF10A cells. These cells grew more rapidly and formed larger but normally polarized acini in Matrigel. Present studies are aimed at delineating the mechanistic basis for these morphogenetic effects.</p>				
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Introduction

The growth, differentiation, and death of normal mammary epithelial cells (MECs) *in vivo* and *in vitro* is dynamically and reciprocally regulated by adhesive interactions with molecules of a basement membrane extracellular matrix (ECM) and to neighboring cells and alterations in the structure and dynamics of these interactions is associated with and necessary to neoplastic progression. Of the many levels of regulation the state of tyrosine phosphorylation of structural and regulatory proteins linked to adhesive interactions by the opposing actions of tyrosine kinases (TKs) and protein tyrosine phosphatases (PTPs) is linked to the dynamics of (dis)assembly of, and signaling from these structures. While the roles of TKs in these processes have been more thoroughly investigated those of PTPs are much less well understood. Of the three major classes of the PTP superfamily now recognized, many of the "classical" transmembrane and non-transmembrane PTPs have extra-catalytic structural features that, borne out by emerging functional studies, suggest roles either as cell-ECM or cell-cell adhesion receptors in their own right or in association with well characterized adhesion linked intra- and extracellular structural complexes and proteins and are good candidates as regulators of adhesive interactions in normal and malignant MECs. In this project we have proposed and begun to characterize the expression, and regulatory and functional relationships between this class of PTPs in general and of the highly expressed PTPs, cytosolic PTP-PEST and transmembrane RPTP- κ in particular, and cellular adhesion in an *in vitro* model of mammary tumorigenic progression; the HMT-3522 cell series. Representative passages from this series recapitulate some of the phenotypic range characteristic of breast cancer progression *in vivo*, particularly in their growth patterns embedded in a laminin-rich exogenous basement membrane preparation (MatrigelTM) (3D BM assay) that are functionally linked to changes in expression of integrin-ECM adhesion receptors. Understanding the details of the structure and regulation of adhesive interactions by PTPs and their relationship to cell fate decisions will lead to improvements in prognostic, diagnostic and therapeutic approaches to breast cancer.

This final report is addressed to the Specific Aims/Statement of Work of the original proposal I submitted in in FY2000 as relates to the protein tyrosine phosphatase (PTP) PEST (PTPN12). I state at the outset that although I proposed to also characterize in detail the transmembrane protein RPTPK (κ) this was ambitious and little work has been done on this protein. However, the lessons learned from the former are proving to be useful in designing approaches for this and other PTPs (and other proteins) in relation to mammary epithelial cells (MECs) our lab is currently investigating. On the other hand I note that we have expanded beyond the original proposal in relation to the approach to PTPN12, including both stable RNA interference (which is yielding some exciting results) and EGFP labeled PTPN12 derivatives that will be useful in characterizing the cellular localization, regulation, and dynamics of a completely unexpected but exciting observation; the localization of PTPN12 to the mitotic spindle and (cleavage furrow) plasma membrane in metaphase cells. Publications creditable to the CDMR-BCRP are certainly forthcoming and I will be working over the next year in Dr. Weaver's lab towards their completion. I wish to express my thanks to the DOD for their support for this research.

Body

A. Stable Recombinant Expression of Wild-type and Dominant Negative/Substrate Trapping Versions of PTP-PEST and RNA interference of PTPN12 in Human MECs

Under Task 1 and Task 3 of the Statement of Work I proposed to generate wild type, dominant negative, and substrate trapping expression constructs of human PTPN12 and RPTPK in the tetracycline inducible HERMES pHRSpuro retroviral vector (RetroTet-ART system, generous gift of Dr. Helen Blau, Stanford University) [1] (Figure 1) and use these to stably transduce representative passages of the HMT 3522 cell series including non-malignant S-1, premalignant S-2 and tumorigenic T4-2. With

respect to PTPN12 this has been accomplished. Additional constructs have also been generated beyond those outlined in the Statement of Work. These include carboxy- and amino- terminal EGFP fusion constructs with PTPN12 (and mutant derivatives), bicistronic (via an internal ribosome entry site; IRES) EGFP versions of HERMES pHRSpuro (Figure 1) and retroviral constructs for the stable introduction of a PTPN12 interfering RNAi (Figure 1).

In the first annual report I described in detail the challenges I encountered in introducing the RetroTet-ART expression system specifically, and retroviral mediated gene delivery in general, in the Weaver laboratory. Retroviral mediated gene delivery continues to be an important methodological approach to the study of mammary epithelial biology in the Weaver laboratory (and others) and I have been at the forefront in introducing further options that other members of the laboratory are using to their advantage. I summarize in brief some of the major challenges/innovations/modifications.

1. Efficient introduction of tetracycline-regulated transactivators into cell lines transduced to puromycin resistance by HERMES pHRSpuro retroviruses. The sensitivity of the RetroTet-ART system to study the biochemical, molecular, and cell biological effects of ectopic expression of proteins depends on the ability to produce titers of recombinant retroviruses expressing these transactivators that achieve transduction efficiencies as near to 100% as possible. For uncertain reasons I was not able to obtain tet-regulated expression of a transgene using the retroviral transactivator (rTab+)/transrepressor (TRg-)(ref) constructs provided with the RetroTet-ART system. However, a fortuitous turn of events in an unsuccessful attempt to simplify the RetroTet-ART system by generating a bicistronic retrovirus expressing both the transactivator rTab+ and TRg- resulted in homologous recombination between rTab+ and TRg- generating a recombinant retrovirus that expressed a tet-off, tet operator binding transactivator that when transactivated into the 293GPG packaging cell line [2] (Generous gift of Dr. Richard Mulligan, MIT) produced a titer that approached 100% transduction efficiency in HMT-3522 MECs (Figure 2). Some difficulties do remain with this modified system. No transrepressor is used so that there is some basal expression of the transgene, but for the genes that we have used to date this appears to be for the most part tolerated.

2. Overcoming poor viral titers. Sufficiently high viral titers are required to achieve use of early passage sufficiently polyclonal populations of retrovirally-transduced cells to prevent population drift in the face

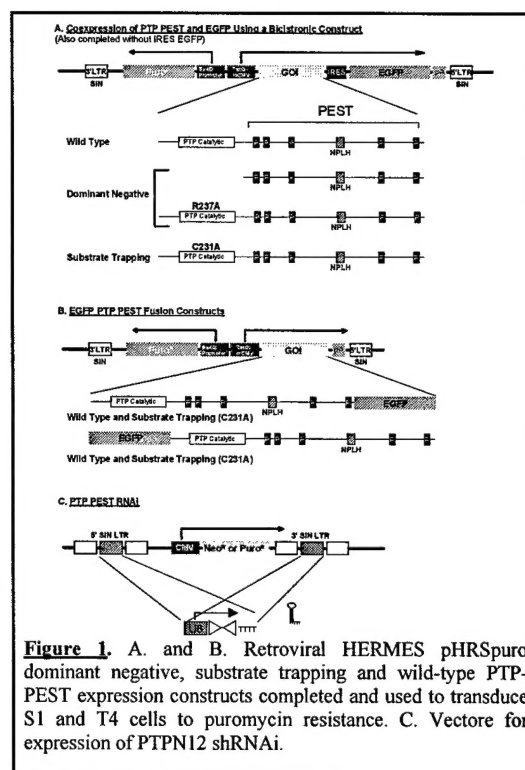


Figure 1. A. and B. Retroviral HERMES pHRSpuro dominant negative, substrate trapping and wild-type PTP-PEST expression constructs completed and used to transduce S1 and T4 cells to puromycin resistance. C. Vector for expression of PTPN12 shRNAi.

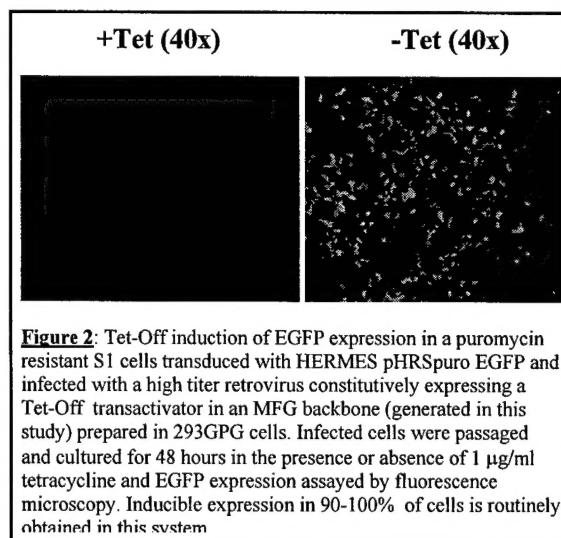


Figure 2: Tet-Off induction of EGFP expression in a puromycin resistant S1 cells transduced with HERMES pHRSpuro EGFP and infected with a high titer retrovirus constitutively expressing a Tet-Off transactivator in an MFG backbone (generated in this study) prepared in 293GPG cells. Infected cells were passaged and cultured for 48 hours in the presence or absence of 1 µg/ml tetracycline and EGFP expression assayed by fluorescence microscopy. Inducible expression in 90-100% of cells is routinely obtained in this system

of the potential secondary effects of retroviral insertional mutagenesis that can lead to the false attribution of observed phenotypic effects to transgene expression. The self-inactivating retroviral vector HERMES pHRSpuro produced generally low titers in a number of common packaging cell systems tried; including 293GPG, Phoenix Ampho (Dr. Gary Nolan, Stanford University), and triple transfection in HEK-293 with vesicular stomatitis virus G (VSVG) protein and gag-pol expressing plasmids (Generous gift of Dr. Alan Kingsman, University of Oxford, Oxford, UK). Acceptable titers of the EGFP expressing control retrovirus were achievable from HERMES pHRSpuro EGFP obtained from Dr. Helen Blau. However, very poor titers were obtained from the corresponding PTPN12 constructs. This was overcome by a combination of approaches. I prepared versions of HERMES pHRSpuro that replace the 5' long terminal repeat (LTR) promoter with a hybrid cytomegalovirus immediate early CMV 5'LTR promoter which is more active in HEK-293 cells due to the transactivating effects of transforming adenoviral E1A protein in these cells. In addition, a BspHI fragment containing the recombinant proviral construct can be ligated to a BspHI fragment from the plzrs vector (Generous gift of Dr. Gary Nolan, as described at <http://www.stanford.edu/group/nolan>) containing an expression cassette for the Epstein Barr Virus Nuclear Antigen-1 (EBNA-1)/EBV origin of replication (OriP) and an expression cassette for the puromycin resistance gene; features allowing selection of stable episomal replication in 293 and derivative (including Phoenix Ampho) cell lines. These latter constructs improved titers both in transient triple transfection protocols in 293 cells and allowed the rapid production of polyclonal high titer producer lines in Phoenix ampho cells.

Retroviral mediated stable downregulation of PTPN12 via a short-hairpin interfering RNA (shRNAi)

Since submission of my proposal in FY2000 a flurry of reports have detailed the downregulation of cognate mammalian mRNAs by short 19-29 bp RNA duplexes, and the stable mediation of this by

RNA polIII promoters (ex. U6 snRNA), expressing similar short RNA stem-loops, incorporated in retroviruses [3]. Although not covered by my original proposal I decided that a useful complementary approach to understanding the role of PTPs in MECs would be their stable downregulation by RNAi. To this end three such shRNA's corresponding to 19 nt sequences of the non-catalytic domain of human PTPN12 were designed using the siDESIGN software (<http://www.dharmacon.com/>) and used to prepare shRNA constructs driven by a U6 promoter and incorporated in the 3' LTR of a SIN retroviral vector (Figure 1). One of these U6 shRNA constructs was effective at inhibiting expression of an EGFP-PTPN12 fusion expression construct in cotransfected 293 cells (Data not shown). A retroviral construct with this U6 shRNA was used to transduce MECs and has been shown by semi-quantitative RT-PCR (developed under Specific Aim 2) to downregulate PTPN12 at the mRNA level by an estimated 70 to 80% (Figure 3A and B). The phenotype of transduced MECs as it is presently understood will be discussed later in this report.

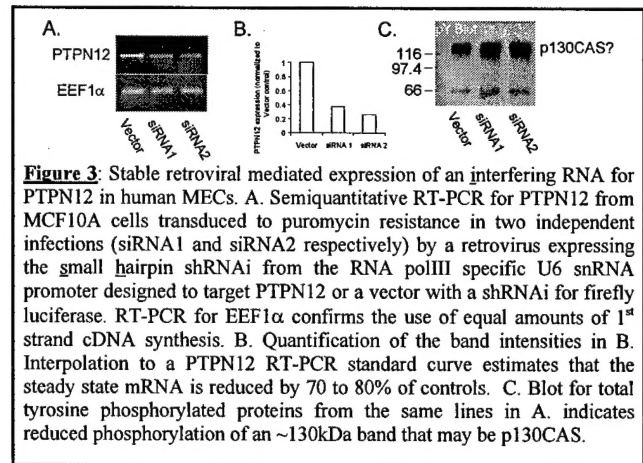
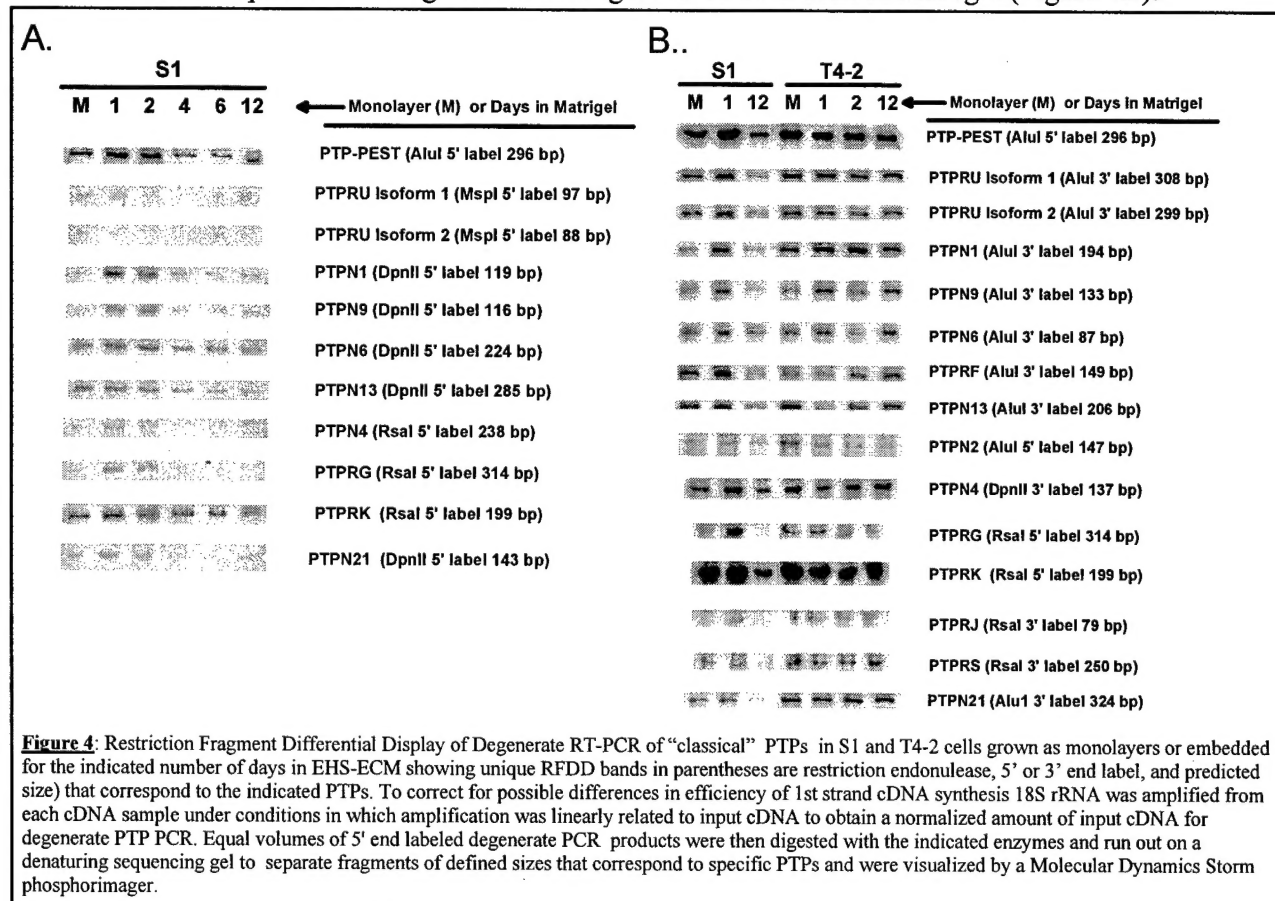


Figure 3: Stable retroviral mediated expression of an interfering RNA for PTPN12 in human MECs. A. Semiquantitative RT-PCR for PTPN12 from MCF10A cells transduced to puromycin resistance in two independent infections (siRNA1 and siRNA2 respectively) by a retrovirus expressing the small hairpin shRNAi from the RNA polIII specific U6 snRNA promoter designed to target PTPN12 or a vector with a shRNAi for firefly luciferase. RT-PCR for EEf1α confirms the use of equal amounts of 1st strand cDNA synthesis. B. Quantification of the band intensities in B. Interpolation to a PTPN12 RT-PCR standard curve estimates that the steady state mRNA is reduced by 70 to 80% of controls. C. Blot for total tyrosine phosphorylated proteins from the same lines in A. indicates reduced phosphorylation of an ~130kDa band that may be p130CAS.

B. Characterization of the expression and localization of PTPN12 during normal and dysregulated morphogenesis of HMT-3522 cells in EHS-ECM

I. Regulation of expression of PTPN12 and other PTPs in MEC cell lines

A combination of restriction fragment differential display (RFDD) of degenerate (PTP catalytic domain specific) PCR, sequencing of cloned degenerate PCR amplified PTP catalytic domains, specific PCR, and (oligonucleotide; Invitrogen) microarray expression analysis was used to identify a list of 21 likely expressed "classical" PTPs in HMT-3522 MECs (Table 1) including a previously unreported isoform (isoform 2, Table 1) of receptor PTPRU. The latter features 9 bp (presumptively) spliced out of the catalytic domain (ie maintaining the reading frame) of the 3 isoforms listed in Locuslink (I have since verified that this isoform is also found in another independently isolated human MEC cell line; MCF10A). Identification of unique RFDD restriction fragments allowed use of this technique to determine in parallel the likely expression of "classical" PTPs in a number of total RNA samples simultaneously; in particular in S1 and T4-2 HMT-3522 MECs growing in 2D monolayer culture and undergoing normal or dysregulated morphogenesis, respectively embedded in EHS-ECM (Matrigel™) (Figure 4). None of the PTPs observed in this analysis were uniquely expressed in normal, S1 or tumorigenic, T4-2 cells. However, a number appeared to be expressed at similar or higher levels in T4-2 cells than in S1 cells in nearly all conditions except in the first 2 days after embedding in Matrigel (Figure 4, A. and B.). In S1 cells, while some PTPs are expressed at near constant levels, a number are transiently upregulated (with somewhat different degrees and kinetics of induction) early in morphogenesis (days 1 and 2) and then decrease by day 4 reaching levels either similar or lower than monolayer by day 12. This is similar in many respects to the expression of the orthologous PTPs in the mouse mammary gland, being elevated during remodeling associated with pregnancy and downregulated in the fully remodeled lactational gland [4]. By contrast there was less obviously any variation of PTP expression during "tumor-like" growth of T4-2 cells in Matrigel (Figure 4B).



PTP (Locuslink)	Synonyms (From Locuslink)	Amplified PTP Domain (N or C Terminal)	Molecular Wt (nts) of Expected RFDD Band with 5' or 3' Primer End Labeled								Intensity of RFDD Band
			Alul (3')	Alul (5')	DpnII (3')	DpnII (5')	MspI (3')	MspI (5')	RsaI (3')	RsaI (5')	
PTPN12	PTP-PEST, PTPG1	N/A	72 (Y)	296 (Y)	329 (Y)	35 (Y)	None	None	None	None	(++)
PTPN13	FAP1, PTP1E, PTP-BAS, PTPL1	N/A	206 (Y)	165 (Y)	42 (Y)	285 (Y)	None	None	None	None	(++)
PTPN21	PTPD1, PTPRL10	N/A	324 (Y)	83 (Y)	55 (Y)	143 (Y)	190 (Y)	153 (Y)	316 (Y)	91 (Y)	(++)
PTPN1	PTP1B, PTP-1B	N/A	194 (Y)	192 (Y)	152 (Y)	119 (Y)	47 (Y)	337 (Y)	None	None	(++)
PTPN6	HCP, HCPH, SHP-1, PTP-1C, SHP-1L	N/A	87 (Y)	248 (Y)	71 (Y)	224 (Y)	None	72 (Y)	139 (Y)	180 (Y)	(++)
PTPN9	MEG2	N/A	133 (Y)	248 (Y)	293 (Y)	116 (Y)	None	None	322 (Y)	91 (Y)	(++)
PTPN4	PTPMEG	N/A	None	None	137 (Y)	230 (Y)	None	None	65 (Y)	238 (Y)	(++)
PTPN2	PTPT, Tcell PTP	N/A	100 (Y)	147 (Y)	26	353	None	None	201 (Y)	91 (Y)	(+)
PTPRU	PI, FMI, PTP, PCF-2, PTP-J, PTPRO, PTPU2, GLEPP1, HPTP, J, PTP-PI, R- PTP-ESI	N (Isoform 1)	308 (Y)	57 (Y)	314 (Y)	47 (Y)	40 (Y)	97 (Y)	273 (Y)	91 (Y)	(++)
		N (Isoform 2)	299 (Y)	57 (Y)	305 (Y)	47 (Y)	40 (Y)	88 (Y)	273 (Y)	82 (Y)	(++)
		C	226	57	None	None	117	111	289	94	
PTPRA	LRP, HLPR, PTPA, HPTPA, RTPA, PTPRL2 (alpha)	N	126	254	92	284	None	None	207	91	
		C	207 (Y)	164 (Y)	86 (Y)	218 (Y)	147 (Y)	213 (Y)	280 (Y)	91 (Y)	(+)
PTPRK	R-PTP-kappa	N	None	None	None	None	72 (Y)	72 (Y)	27	199 (Y)	(++)
		C	None	None	108	161	36	168	145	33	
PTPRG	PTPG, HPTPG, RTPPG, D3S1249 (gamma)	N	58 (Y)	343 (Y)	66 (Y)	84 (Y)	None	None	86 (Y)	314 (Y)	(+)
		C	162	65	331	35	None	None	82	88	
PTPRS	(sigma)	N	212	156	360	74	41	247	None	None	
		C	317 (Y)	57 (Y)	355	35 (Y)	125 (Y)	72 (Y)	250 (Y)	91 (Y)	(+)
PTPRF	LAR	N	149 (Y)	156 (Y)	280 (Y)	84 (Y)	294 (Y)	72 (Y)	100 (Y)	91 (Y)	(+)
		C	317	57	None	None	150	198	359	91	
PTPRJ	DEP1	N/A	None	None	None	None	81 (Y)	160 (Y)	79 (Y)	295 (Y)	(+/-)

Table 1: Summary table of "classical" PTPs expressed in cells of the human HMT-3552 cell progression series. Shown under RFDD are the length of fragments expected for 5' or 3' labeled degenerate RT-PCR of total PTPs for the indicated restriction enzymes for the given PTP. In parenthesis after these sizes "Y" indicates that a band of this size was observed on RFDD gels. Red indicates that the fragment size is unique for the given PTP among the list of detected PTPs and therefore could be used reliably for quantitation across biological samples. Expected bands for all PTPs in blue based on predictions from the sequences in Genbank were reliably observed in RFDD analyses giving high confidence for the identification. Those in white were not observed in the RFDD analysis. Additional PTPs whose expression in HMT3522 MECs has been verified by RT-PCR, Western Blot, and/or microarray expression analysis include PTPN3, PTPN11, PTPRZ1, PTPRJ, PTPRM, PTPRM, PTPRB, and PTPRE.

This work has proceeded on two fronts. First, I have developed a specific (relative) quantitative RT-PCR assay for several PTPs to verify observations made using RFDD of degenerate PCR products and established conditions for effective normalization using *EEF1α* (selected by microarray analysis as an abundant mRNA varying little across the HMT3522 cell series) as an invariant control. Figure 5 shows in outline this approach. Densitometric analysis of 28S and 18S rRNA bands of unknown samples of total RNA on ethidium bromide stained denaturing agarose gels are interpolated to a standard curve of total RNA to obtain a normalized input total RNA for 1st strand cDNA synthesis (Figure 5A). 1st strand cDNA syntheses are subjected to PCR for *EEF1α* under conditions selected to yield a linear relationship between product yield and input cDNA of a set of dilutions of a standard cDNA sample (Figure 5B) run in parallel and unknowns are interpolated to this curve to obtain a relative normalization

factor for input cDNA. Equivalent reamplification of unknowns for *EEF1α* based on these relative normalization factors confirms the validity of these relative normalization factors (Figure 5C). These factors are then used to normalize input cDNA for specific PCR of selected PTPs which is either done as semi-quantitatively or (relatively) quantitative by developing similar standard curves for specific PTPs. Figure 5D shows this as a semi-quantitative PCR for PTPN12, 13, and 4 using specific primers that I designed using Primer3 software available online from the Whitehead Institute (Cambridge, MA) in S1 cells as monolayer or for the indicated number of days after embedding in Matrigel confirming the quantitative interpretation of the RFDD analysis (cf. Figure 4A and Figure 5D with respect to these genes).

Second, operating under the hypothesis that regulated or transient expression of PTPs in S1 cells growing in Matrigel and in the pregnancy/lactational development of the mouse mammary gland indicate participation in, and/or are reflective of an orderly morphogenetic progression that is lost in tumorigenic cells, I have been interested in trying to identify some possible signals/conditions that affect PTP expression in normal MECs. Many of these "classical" PTPs have extra-catalytic domains and/or have been functionally linked to regulation of cell and tissue structure via the cytoskeleton and/or cell-cell and cell-ECM interactions suggesting that control of expression may reflect signals relating to these events. One possibility is that variation of expression is related to cellular proliferation; high in proliferative S1 cells when first embedded in Matrigel and decreasing as cells growth arrest at later times. While I cannot eliminate the possibility that at least some (or most for some PTPs) regulation of expression is simply related to this, a kinetic analysis of growth rates of S1 spheroids in Matrigel (spheroid size as nuclei per spheroid and Ki67 +ve nuclei per spheroid, Figure 6) suggests that by day 4 when expression of some PTPs (ex PTPN4, PTPRG, PTPN21) is observed to decrease sharply most S1 cells still appear to be actively growing (indicated by near concordance of nuclei/spheroid and Ki67+ve nuclei/spheroid, Figure 6).

Since down-regulation of PTP expression occurs in the transition from an average of 2 to an average of 8 cell clusters (Figure 6) one possibility is that down-regulation may be linked in part to formation of adherens junctions (AJ) between cells and or an increase in cell-cell in relation to cell ECM interactions. In addition higher levels of expression of PTPs in T4 cells may correlate with derangements of AJ formation as is seen with many epithelial cancers. To test the idea that there is a feedback regulation of the dynamics of adhesion structures important for normal MEC morphogenesis on PTP expression I previously reported on the preparation of S1 cells stably transduced with HERMES pHRSpuro retroviruses that inducibly express dominant negative versions of E-cadherin (Murine MHC

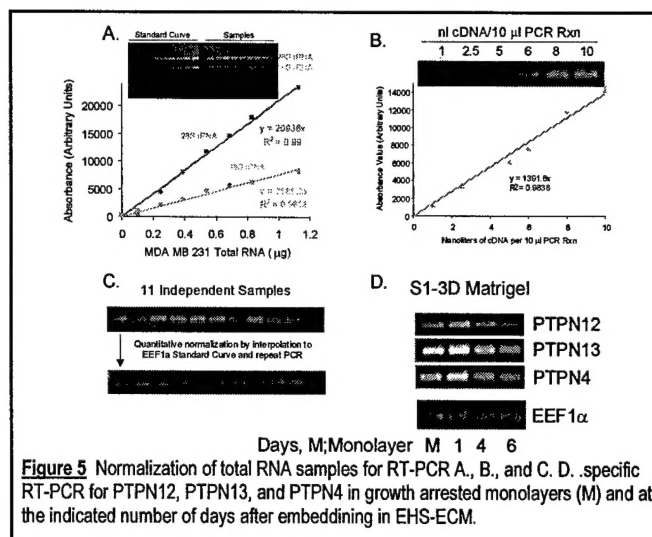


Figure 5 Normalization of total RNA samples for RT-PCR. A., B., and C. D. specific RT-PCR for PTPN12, PTPN13, and PTPN4 in growth arrested monolayers (M) and at the indicated number of days after embedding in EHS-ECM.

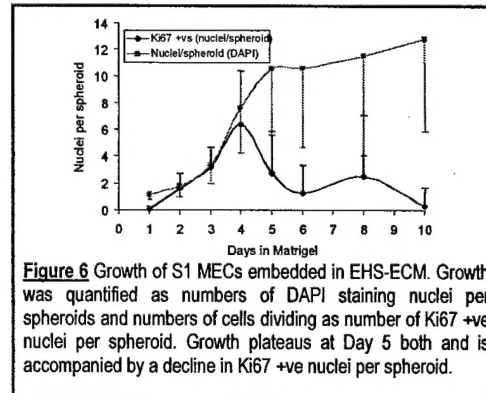


Figure 6 Growth of S1 MECs embedded in EHS-ECM. Growth was quantified as numbers of DAPI staining nuclei per spheroids and numbers of cells dividing as number of Ki67 +ve nuclei per spheroid. Growth plateaus at Day 5 both and is accompanied by a decline in Ki67 +ve nuclei per spheroid.

Class I H-2K^d extracellular domain fused to transmembrane and cytoplasmic domains of human E-cadherin) [5]. Expression of this mutant was linked to coexpression of EGFP from a bicistronic mRNA to correlate phenotype with EGFP expression. As shown in Figure 7A (Ecad cyto) expression of this construct in S1 MECs in monolayer cultures resulted in a scattered disaggregated morphology in a majority of cells correlated nearly 1 to 1 with EGFP expression

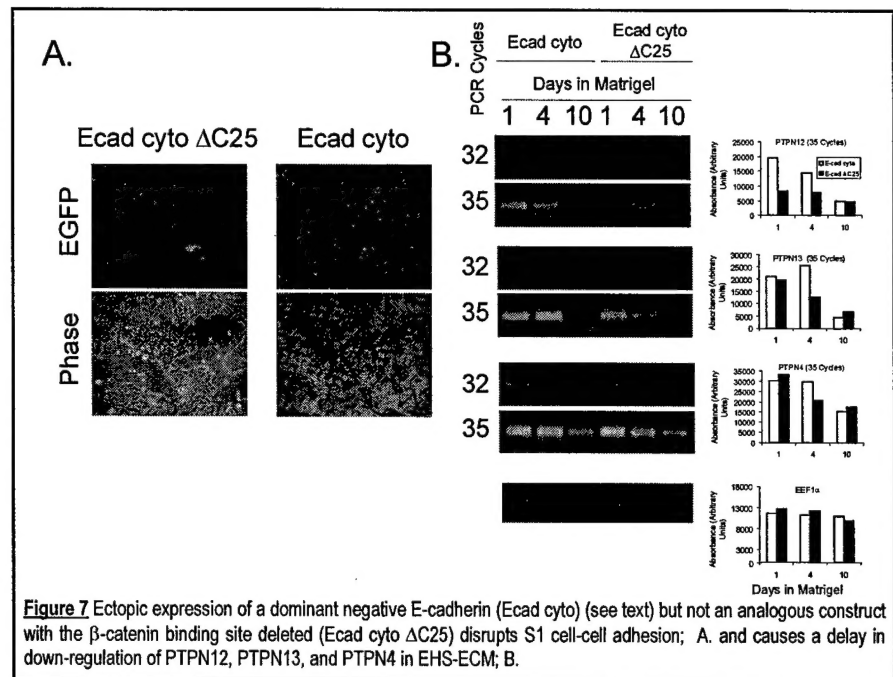


Figure 7 Ectopic expression of a dominant negative E-cadherin (Ecad cyto) (see text) but not an analogous construct with the β -catenin binding site deleted (Ecad cyto Δ C25) disrupts S1 cell-cell adhesion; A. and causes a delay in down-regulation of PTPN12, PTPN13, and PTPN4 in EHS-ECM; B.

consistent with inhibition of adherens junction (AJ) formation. In contrast a control construct in which the cytoplasmic β -catenin binding site was deleted (Ecad cyto Δ C25) maintained normal MEC morphology. In Matrigel cultures dominant negative but not control transduced S1 cells grew as grape-like clusters as opposed to the tight spherical clusters of normal S1 3D cultures (Data Not Shown). Semi-quantitative RT-PCR of PTPN12, PTPN13, and PTPN4 of such transduced S1 cells grown for the indicated times in Matrigel showed that at Day 1 only PTPN12 (PEST) was up-regulated in Ecad cyto cells relative to the control (Figure 6B). However, all three PTPs maintained an elevated expression in Ecad cyto cells at Day 4 relative to control although all appeared to be down-regulated to control levels by Day 10. This delay in down-regulation is consistent with a direct or indirect feedback role for AJ formation on PTP expression. However the decrease of PTP expression to control levels by Day 10, in spite of the presence of a significant number of EGFP positive grape-like clusters in Day 10 cultures complicates a straightforward interpretation. One likely possibility is that downregulation of PTP expression is subject to many levels of regulation and in late S1 cultures is more directly related to growth arrest *per se* since these cultures appear to growth arrest despite the inhibition of cell-cell adhesion, an observation that is consistent with the attenuation of proliferation and enhanced differentiation of keratinocytes by overexpression of ecad cyto [5].

Recently, I have obtained preliminary evidence suggesting that PTP expression may also reflect at some level the mechanical properties of the cellular microenvironment. Of the many variables that change when MECs are grown on tissue culture plastic as opposed to embedded in Matrigel or as part of a tissue is the mechanical compliance of the substrata to which they are adhering, Matrigel being "soft" relative to "stiff" tissue culture plastic. By varying the concentration of total acrylamide, and of acrylamide to bisacrylamide it is possible to generate a range of linearly elastic gels of widely varying compliance [6]. By incorporating a N-succidymal ester linked to an acrylyl group into these gels during polymerization a derivatized gel is prepared to which proteins can be subsequently crosslinked via mild and facile chemistry [7]. Using this approach we have been able to cross link Matrigel proteins to polyacrylamide gels that vary from "soft" (normal-tissue-like; Elastic modulus, $E = 1250$ Pa) to "stiff" ($E = 60000$ Pa) separating as independent variables the compliancy of the matrix from its biochemical

signaling properties. When the normal human MEC cell line MCF10A are plated on these gels for 24 hr they attach and spread to varying extents (on average less spread on "soft" gels, Figure 8A). Studies in our laboratory have shown that MCF10A cells on stiff gels have larger and more numerous vinculin positive focal adhesions, and increases in actin stress fibers and are at the biochemical level by increases in phosphorylation of Y397 FAK and in the total and activated (tyrosine phosphorylated) forms of src family kinases, lyn and lck (N. Zahir, Unpublished observations). Figure 8B shows the differential ratio of expression of selected PTPs observed by RFDD analysis of total RNA isolated from

MCF10A cells on "soft" vs "stiff" gels. The variation in expression indicates relative ratios varying by at most 2 fold for those PTPs in this preliminary analysis with some unchanged (PTPN1, PTPN9, PTPN13) some higher on "stiff" gels (2 fold for PTPN6, PTPRS, PTPRF, PTPRU) and two modestly higher on "soft" gels (1.4 fold PTPN12, and 1.8 fold for PTPN21). Preliminary semi-quantitative RT-PCR for PTPN12 appears to confirm that it is upregulated on "soft" matrices (Figure 8C) and in this analysis by possibly more than was suggested by RFDD (~2.5 fold difference in band intensities). In PTPN12 null mouse embryo fibroblasts focal adhesion proteins FAK, p130CAS, and paxillin are hyperphosphorylated [8, 9]. Increases in expression of PTPN12 in response to increases in matrix compliancy may be in part responsible directly or indirectly for the observed hypophosphorylation of Y397FAK. This observation is interesting in light of the apparent differential growth effects of PTPN12 RNAi on MCF10A cells on "stiff" (plastic) and "soft" (Matrigel) substrata. Additionally, since changes in the organization of the actin cytoskeleton are one of the marked morphological responses of cells to increasing compliancy it is of interest to note that the three PTPs (PTPN12, and Band 4.1 domain containing PTPN4, and PTPN21) apparently upregulated on soft gels are all implicated to varying degrees in its regulation.

II. Localization of PTPN12 in Human MECs

I obtained an antibody to human PTPN12 (Generous gift of N.Tonks) to be used in localizing PTPN12 in human MECs growing on tissue culture plastic and in cells growing on EHS-ECM. PTPN12 was diffusely cytoplasmic in MECs grown both on tissue culture plastic and in EHS-ECM, as previously reported (Figure 9A and B). In subconfluent cells on tissue culture plastic occasional cells were observed in which PTPN12 was observed at the leading edge of lamellopodia colocalizing with other proteins of the leading edge (p190-B RhoGAP in Figure 9C) where it is presumed to regulate focal adhesions and Rho family GTPases. In human MECs in EHS-ECM, which do not form distinct focal adhesions no such distinct immunostaining was observed, although there was a pronounced perinuclear staining (arrowhead, Figure 9A). To verify these observations I have also prepared EGFP N- and C-terminally tagged PTPN12 and used retroviral mediated gene transfer to introduce these into human

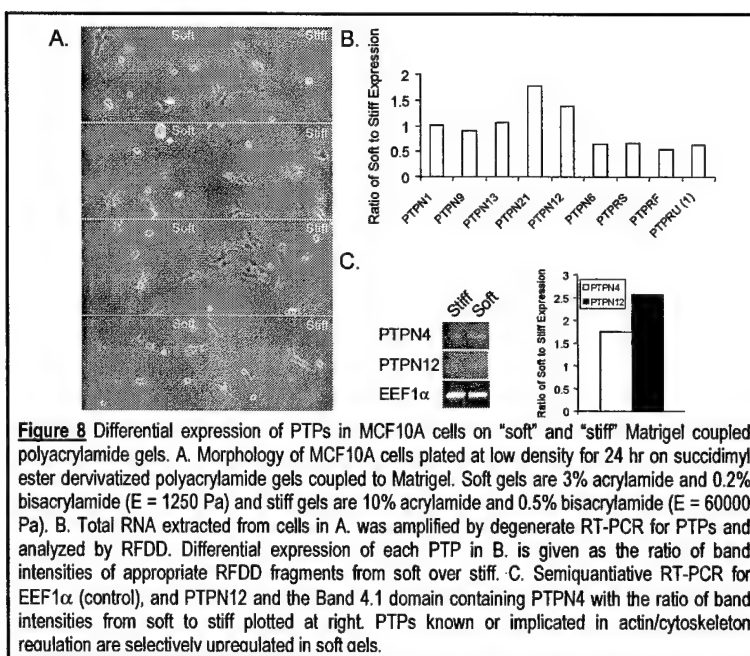
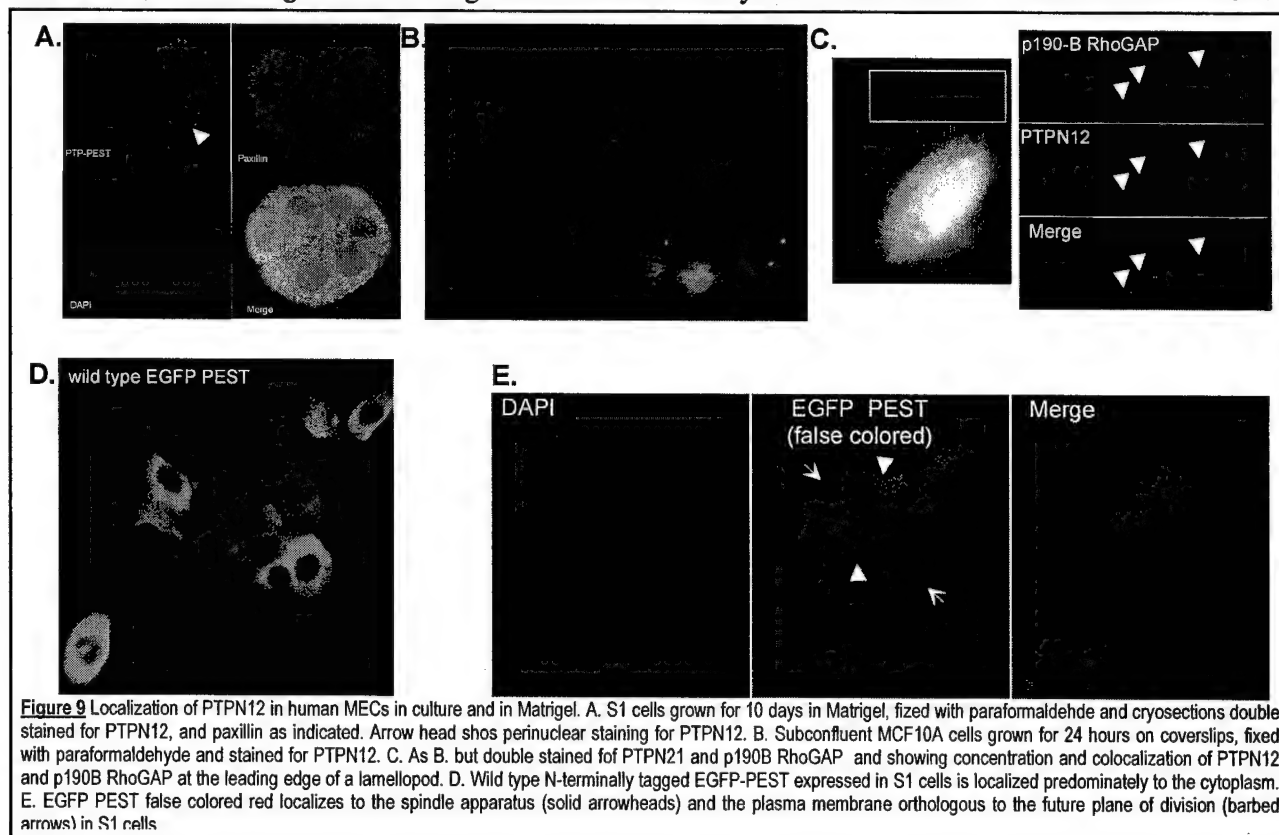


Figure 8 Differential expression of PTPs in MCF10A cells on "soft" and "stiff" Matrigel coupled polyacrylamide gels. A. Morphology of MCF10A cells plated at low density for 24 hr on succinyl ester derivatized polyacrylamide gels coupled to Matrigel. Soft gels are 3% acrylamide and 0.2% bisacrylamide (E = 1250 Pa) and stiff gels are 10% acrylamide and 0.5% bisacrylamide (E = 60000 Pa). B. Total RNA extracted from cells in A. was amplified by degenerate RT-PCR for PTPs and analyzed by RFDD. Differential expression of each PTP in B. is given as the ratio of band intensities of appropriate RFDD fragments from soft over stiff. C. Semi-quantitative RT-PCR for EEf1α (control), and PTPN12 and the Band 4.1 domain containing PTPN4 with the ratio of band intensities from soft to stiff plotted at right. PTPs known or implicated in actin/cytoskeleton regulation are selectively upregulated in soft gels.

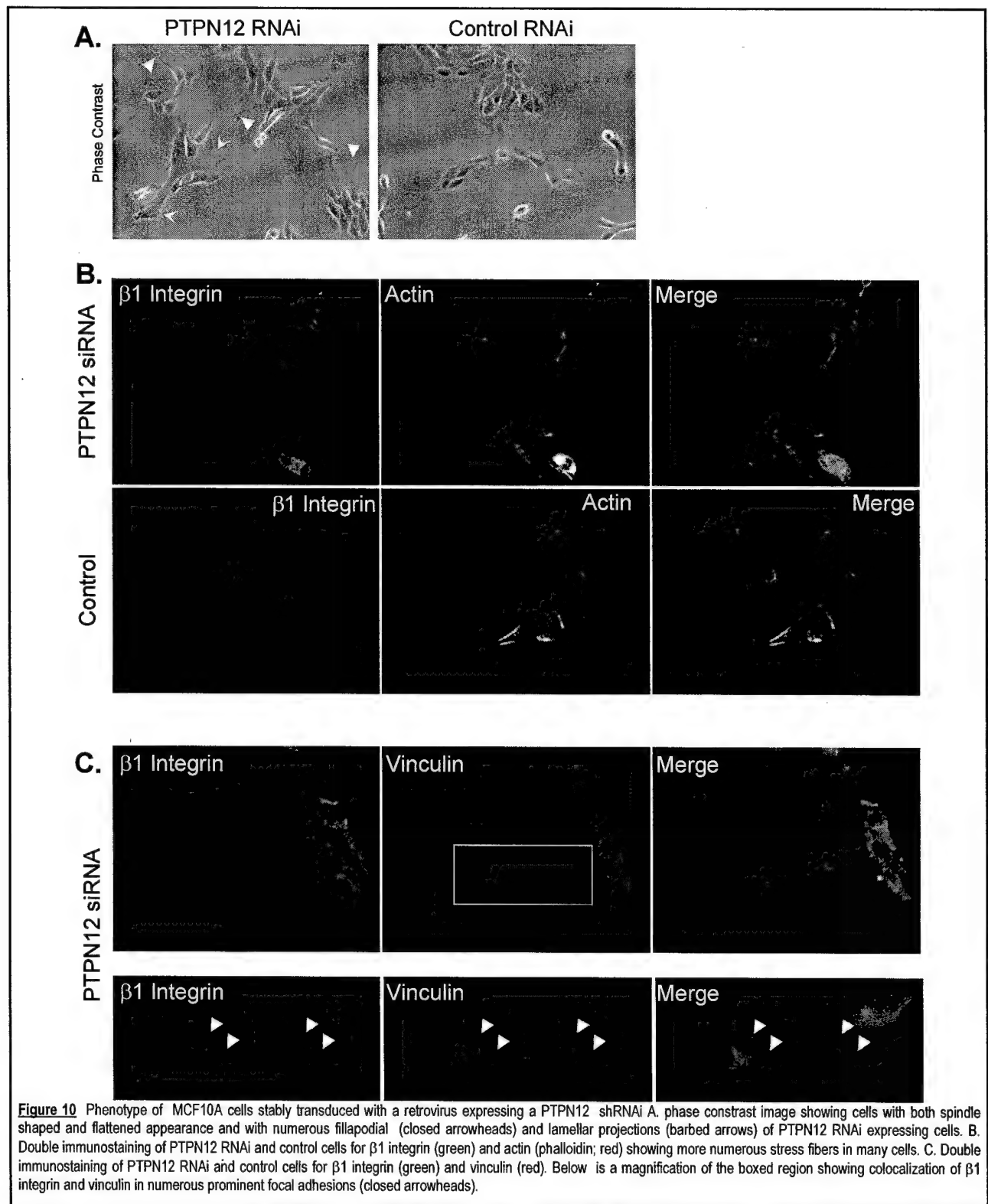
MECs. The C-terminally tagged proteins were not as bright as those tagged at the N-terminus. PTPN12 tagged at the N terminus verified a cytoplasmic localization (Figure 9D and E). Interestingly, I occasionally identified metaphase cells that displayed a surprising and to my knowledge unreported localization. EGFP-PTPN12 could be seen lighting up the spindle apparatus (solid arrows, Figure 9E). In addition, in the image shown in Figure 9E one can clearly see a membranous concentration of EGFP-



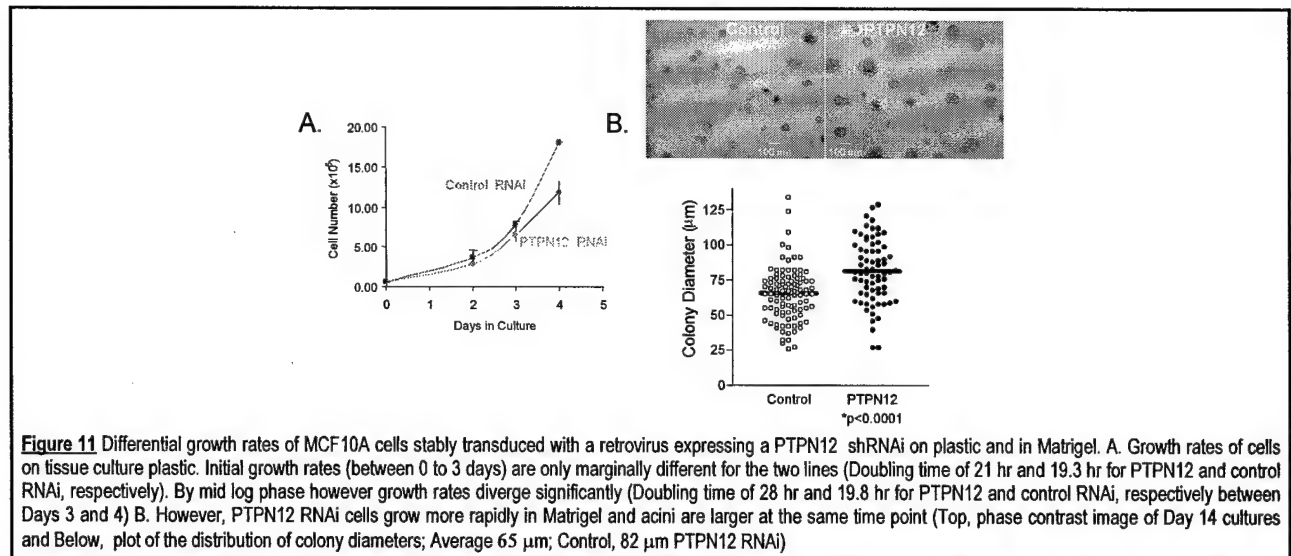
PTPN12 in an arc extending orthogonal to the future plane of division (barbed arrows, Figure 9E) and absent from the membranous regions at the poles of the daughter cells. PTPN12 null fibroblasts exhibited delayed resolution of cytokinesis [9]. Several actin regulating proteins also implicated in cytokinesis, including PSTPIP1 [9], and p190-RhoGAP [10] that interact with PTPN12 have been localized to similar membranous regions in dividing cells potentially providing a basis for this localization. However, localization of neither protein to the spindle apparatus has been reported suggesting that the role for PTPN12 in cell division may be richer than previously appreciated. These observations suggest that EGFP-PTPN12 will be a useful tool for the analysis of the dynamics and role of PTPN12 in cellular division.

C. Characterization of the Molecular and Cellular Effects of Perturbation of PTPN12 Function in Human MECs and Identification of Impacted Signaling Pathways

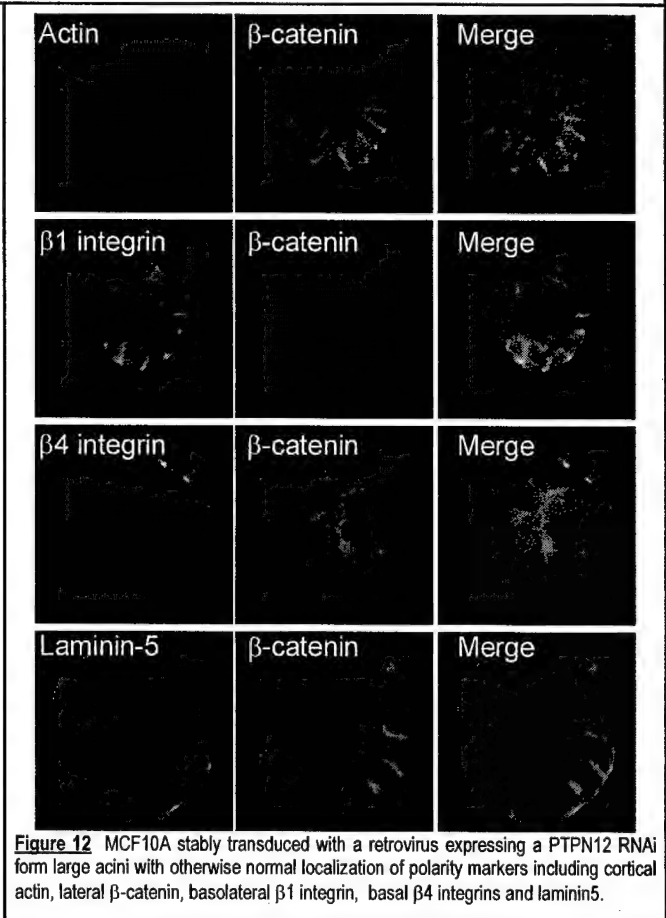
To elucidate the cellular and signaling functions of PTPs in human mammary epithelial cells I proposed the use of tet-inducible stable overexpression of dominant negative versions of PTPN12 in human MECs of the HMT3522 series, S1, S2, and T4-2. As described above I have sought a complementary approach to elucidation of this using retroviral mediated PTPN12 RNAi. Analysis of the cellular effects of stable expression of RNAi in the normal human cell line MCF10A is considerably more mature and will be described. As shown in (Figure 3A and B.) stable expression of a PTPN12 U6



shRNAi induced downregulation of PTPN12 at the mRNA by 70-80% as measured by RT-PCR. Immunoblotting for total phosphotyrosine proteins from these cells indicated that an ~130 kDa pY



protein was most prominently hyperphosphorylated as a consequence of PTPN12 downregulation (Figure 3C.). The size and prominence of this band provide a likely identification with the *crk* associated substrate, p130CAS, previously shown to be a direct substrate of PTPN12 [8, 9, 11]. Some of the cellular effects observed in PTPN12 (-/-) mouse embryo fibroblasts included more rapid spreading, increased stress fibers, larger more mature focal adhesions, and delayed separation of daughter cells through effects on cytokinesis [8, 9, 12, 13]. I found that PTPN12 RNAi MCF10A cells had an unusual morphology including both broad flat cells and more spindle shaped cells (Figure 10A) with numerous unusual filopodial (closed arrowheads, Figure 10A) and broad lamellopodial membrane protrusions (barbed arrows, Figure 10A), had more actin stress fibers (Figure 10B), and larger more numerous and prominent vinculin and $\beta 1$ integrin positive focal adhesions (Figure 10C). In addition, many mitotic cells were observed with long constricted membrane extensions separating daughter cells suggesting a defect in completion of cytokinesis (Data not shown). The growth of PTPN12 RNAi cells is complex.



Somewhat surprisingly on plastic growth rates were not significantly different from controls in early-log phase (Figure 11A., Days 1 to 3, doubling times of 21 hr for PTPN12 RNAi and 19.3 hr for control) indicating importantly that they have the same potential rate of division. However, while control cells maintained the same growth rate in mid-log phase (Days 3 to 4, doubling time of 19.8 hr) PTPN12

RNAi cells began growing markedly slower (doubling time of 28 hr from Days 3 to 4). Paradoxically when these cells were grown embedded in Matrigel PTPN12 RNAi cells grew faster and reached larger overall size (Average 65 μm ; Control, 82 μm PTPN12 RNAi; Figure 11B.). I am at present trying to determine whether these differences represent changes in rates of proliferation and/or apoptosis although the attenuation of growth rates on plastic may be the result of exceeding the carrying capacity of the medium. Another aspect of the phenotype of the PTPN12 RNAi cells is a rapid acidification of the media (readily observable in mid log phase cultures even when the cell density of controls is much higher), which may represent a significant increase in the metabolic rate per cell and presumptively, either a commitment of that energy to purposes (perhaps futile) other than driving growth and proliferation or accumulation of inhibitory waste products. A preliminary analysis indicates that PTPN12 RNAi "acini" in 3D Matrigel are otherwise normal with cleared lumen, cortically organized actin fibers, and normal localization of polarity markers including basal $\beta 4$ integrins and laminin5; lateral β -catenin, and basolateral $\beta 1$ integrin (Figure 12).

D. Future Directions

My intention over the next year is to continue the work supported by this fellowship along two avenues that I anticipate will prove complementary.

1. Regulation of PTP gene expression in normal and malignant human MECs. An intriguing possibility suggested by differential PTP expression in normal MECs growing on soft and stiff Matrigel coupled polyacrylamide gels is that it may reflect the dynamics and state of (actin) cytoskeletal organization. My preliminary evidence suggests that PTPs implicated in the regulation of (actin) cytoskeletal organization may be upregulated on soft gels in association with changes in actin filaments from stress fibers to more cortical networks. It will be important to verify the consistency of these observations by RT-PCR both by expanding the number of PTPs to include those that are unchanged and those that appear to be downregulated on soft gels and the number and type of cells studied, including, in addition to those that are more normal, those that are malignant such as T4s and retain a more stiff-like phenotype on soft gels (M.Paszek, Unpublished observations).

2. Continuation of the characterization of the phenotype of PTPN12 shRNAi expressing MCF10A cells including exploration of signaling pathways impacted. On the basis of previous studies and observations made in this report the molecular effects of shRNAi mediated downregulation of PTPN12 expression appears to be on the regulation and organization of the actin cytoskeleton. Several routes to actin cytoskeleton regulation by PTPN12 have been identified, including activation of Rac1 by dephosphorylation and activation of the Rac1 GEF VAV2 [13, 14] and inactivation of RhoA by dephosphorylation and activation of p190-RhoGAP [14] and inactivation by dephosphorylation of the actin branching Arp2/3 complex activating Wiscott Aldrich Syndrome protein (WASp) [15-17], inhibition by dephosphorylation of c-abl [17], as well as focal adhesion assembly through paxillin [9] or p130CAS [8, 9, 11]. I will determine in shRNAi cells the specific activities of RhoA and Rac1 by pull down assays, and the activation state of the various PTPN12 target by immunoprecipitation and phosphotyrosine immunoblots. Depending upon the outcome of these various approaches I hope to implicate specific pathways in the morphogenetic effects (enhanced growth in Matrigel) of PTPN12 on MCF10A cells and begin to understand these effects in those terms.

Key Research Accomplishments

- Establishment and validation of systems for the stable, rapid, and efficient inducible and constitutive expression of transgenes and short hairpin interfering RNAs in mammary epithelial cells by retroviral mediated gene transfer with transduction efficiencies, and in formats amenable

both to biochemical analyses that should maximize signal-to-noise as well as single cell assays correlating cellular phenotype to transgene expression.

- Preparation of polyclonal puromycin resistant non-malignant S1, MCF10A and tumorigenic T4-2 cell lines transduced with wild-type and dominant negative/substrate trapping mutants of the cytosolic, integrin-linked PTP-PEST in the vector HERMES pHRSpuro for the analysis of this candidate PTP as a regulator of growth and normal and dysregulated morphogenesis.
- Identification and validation of a shRNAi for PTPN12
- Preparation of stable polyclonal populations of non-malignant MCF10A cell lines transduced by a retrovirus expressing an shRNAi for PTPN12. Characterization of the phenotypic consequences of stable PTPN12 downregulation as a means to explore the role of PTPN12 and impacted pathways on morphogenesis.
- Improvement of RT-PCR normalization procedures using $EEF1\alpha$ as control and set up of quantitative and semi quantitative RT-PCR for PTPs including PTPN12, PTPN4, and PTPN13. Verification of expression patterns seen with restriction fragment differential display (RFDD).
- Use of a dominant negative E-cadherin construct expressed stably and inducibly in non malignant S1 cells from a transducing retrovirus and semi quantitative RT-PCR to address the role of adherens junction formation in the downregulation of PTP expression (PTPN12, PTPN13 and PTPN4) during normal morphogenesis of S1 MECs in EHS-ECM.
- Use of low (stiff) and high (soft) compliance Matrigel coupled polyacrylamide gels to explore a role for mechanosensing in regulation of expression of PTPs in human MECs assessed by RFDD and RT-PCR.
- Preparation of PTPN12 tagged at the N-terminus with EGFP to explore the localization, role, and dynamics of PTPN12 in MECs. Use of this in retrovirally transduced MECs to identify a rich role in cytokinesis through a previously unreported localization in metaphase cells to the plasma membrane orthogonal to the plane of division and to the spindle apparatus.

Reportable Outcomes

A. Manuscripts

1. Valerie M. Weaver, Sophie Lelievre, **Johnathon N. Lakins**, Micah A. Chrenek, Johnathon C.R. Jones, Filippo Giancotti, Zena Werb, and Mina J. Bissell; $\beta 4$ Integrin-dependent formation of polarized three-dimensional architecture confers resistance of apoptosis in normal and malignant mammary epithelium. Cancer Cell. 2002 Sep;2(3):205-16.
2. Nastaran Zahir, **Johnathon N. Lakins**, Alan J. Russell, Wenyu Ming, Chandrima Chatterjee, Gabriela Rozenberg, Peter M. Marinkovich, Valerie M. Weaver; Autocrine laminin-5 ligates $\alpha 6 \beta 4$ integrin and activates RAC and NF κ B to mediate anchorage – independent survival of mammary tumors. J Cell Biol, 2003, Dec; 163(6): 1397-407.

B. Abstracts

1. Zahir, N., Ming, W., Russell, A.J., **Lakins, J.N.**, and Weaver, V.M. β 4-integrin-dependent rac activation of NF κ B permits β 1-integrin independent survival in malignant mammary epithelial cells. ASCB 41st annual meeting, Washington D.C, December, 2001
2. **Johnathon N. Lakins**, Micah Chrenek, Baobin Kang, Paul Wong, and Valerie M. Weaver; Adhesion-linked protein tyrosine phosphatases in normal and dysregulated mammary epithelial cell morphogenesis. ASCB 41st annual meeting, Washington D.C, December, 2001
3. Chrenek, M.A., **Lakins, J.N.**, Zahir, N., Wong, P., and Weaver, V.M. Differential Expression of Stress and Apoptosis Related Genes in 3D Culture. ASCB 41st annual meeting, Washington D.C., December 2001.
4. **Johnathon N. Lakins**, Micah Chrenek, Baobin Kang, Paul Wong, and Valerie M. Weaver; Adhesion-linked protein tyrosine phosphatases in normal and dysregulated mammary epithelial cell morphogenesis. Poster #45-6 Presented at DOD Era of Hope BCRP Meeting in September 2002 in Orlando, FL.
5. Zahir, N, Ming, W., Russel, A., **Lakins, J.N.**, and Weaver, V.M. β 4-integrin mediates β 1-integrin independent survival and apoptosis resistance in mammary tumors via activation of rac and NF κ B. Keystone Meeting, 2002.
6. Zahir, N., Ming, W., Russel, A., **Lakins, J.N.**, Marinkovich, P.M., and Weaver, V.M. β 4-integrin mediates mammary tumor survival and apoptosis resistance by inducing IAPs via activation of rac and NF κ B. Mammary Gland Gordon Conference, 2002.
7. Ming W., **Lakins J.N.**, Zahir, N., Kang, B., Unger, M., and Weaver, V.M. β 4-integrin-dependent formation of a polarized 3D tissue structure confers resistance to apoptosis in normal and malignant mammary epithelium via enhanced rac and NF κ B activity. Integrin Gordon Conference, 2002.
8. Zahir, N., Ming, W., **Lakins, J.N.**, Russel, A.J., Chatterjee, C., Marinkovich, M.P., and Weaver, V.M. β 1 integrin independent survival in mammary tumors is linked to upregulation of laminin-5 and β 4 integrin and enhanced activation of rac and NF κ B. ASCB 42nd annual meeting, San Francisco, CA, 2002.
9. Penney M. Gilbert, Meredith A. Unger, **Johnathon N. Lakins**, Nancy Boudreau, Barbara L. Weber, and Valerie M. Weaver; HoxA9 as a putative cell adhesion tumor suppressor (CATS) gene. Chosen for Oral Presentation (Abstract #18) at ASCB December 2002 Annual Meeting in San Francisco, CA.
10. Nastaran Zahir, Wenyu Ming, **Johnathon N. Lakins**, Alan J. Russell, Chandrima Chatterjee, Peter M. Marinkovich, Valerie M. Weaver; β 1 Integrin independent growth and survival in mammary tumors is linked to upregulation of laminin 5 and β 4 integrin

and enhanced activation of Rac and NF κ B. Poster # 365 presented ASCB December 2002 Annual Meeting in San Francisco, CA.

11. Gabriela I. Rozenberg, **Johnathon N. Lakins**, Valerie M. Weaver; The malignant phenotype of mammary epithelial cells is linked to increased expression of fibronectin and $\alpha 5 \beta 1$ integrin. Poster #2734 presented ASCB December 2002 Annual Meeting in San Francisco, CA.
12. Paszek, M.J., Zahir, N., **Lakins, J.N.**, Reinhart-King, C.A., Dembo, M., Janmey, P.A., Hammer, D.A., and Weaver, V.M. Biophysical properties of the extracellular matrix regulate integrins and isometric forces to modify cell phenotype. ASCB 43rd annual meeting, San Francisco, CA, 2003.
13. Brenda Alston-Mills, Baobin Kang, Chandrima Chatterjee, **Johnathon N. Lakins**, Calvin Roskelley, and Valerie M. Weaver; Differential regulation of SAPK/JNK modulates $\alpha 6 \beta 4$ integrin dependent apoptosis resistance. Poster presented at Gordon Conference on Mammary Gland Biology in June 2003 in Providence RI.
14. P.M. Gilbert, **J.N. Lakins**, M.A.Unger, M. Benezra, J.D. Licht, B.L. Weber, N.Boudreau, V.M. Weaver; HOXA9 Suppresses Breast Tumor Behavior in Association With Modulation of BRCA1 Expression. Poster Presentation at ASCB, San Francisco, CA, 2003
15. Zahir, N., Johnson, K.R., **Lakins, J.N.**, Paszek, M., Gasser, D., Margulies, S.S. and Weaver, V.M. Spatial-Mechanical Regulation of Mammary Epithelial Cell Morphogenesis. BMES Annual Meeting, Philadelphia, PA, 2004
16. Paszek, M., Zahir, N., **Lakins, J.N.**, Lynch, L., Boettiger, D., Hammer, D., and Weaver, V.M. Characterizing Integrin Dependent Force Transduction. BMES Annual Meeting, Philadelphia, PA, 2004.
17. Paszek, M., Zahir, N., **Lakins, J.N.**, Reinhart-King, C.A., Lynch, L., Boettiger, D., Weaver, V.M. and Hammer, D. Molecular Basis of Cellular Traction Force Generation. BMES Annual Meeting, Philadelphia, PA, 2004.
18. Paszek, M., Zahir, N., **Lakins, J.N.**, Johnson, K.R., Rozenberg, G.I., Dembo, M., Boettiger, D., Hammer, D., and Weaver, V.M.; Mechano-signaling in mammary morphogenesis and tumorigenesis Poster Presentation submitted to ASCB, Washington, DC 2004
19. **Lakins, J.N.**, and Weaver, V.M.; RNA interference of PTPN12 (PEST) enhances acinar growth of MCF10A cells potentially through activation of Rho. Poster Presentation submitted to ASCB, Washington, DC 2004

C. Development of Cell Lines

1. Non-malignant S1, MCF10A, and T4-2 cells stably transduced by a retrovirus expressing a PTPN12 shRNAi.
2. Non-malignant S1, MCF10A, and T4-2 cells stably transduced by a retrovirus expressing an N-terminal EGFP- PTPN12 fusion protein.
3. Non-malignant S1, MCF10A, and T4-2 cells stably transduced by a retrovirus enabling inducible expression of wild type and dominant negative/substrate trapping mutants of human PTP-PEST as described in Figure 1.
4. Non-malignant S1 stably transduced by a retrovirus enabling inducible expression of dominant negative mutants of β 4-integrin, E-cadherin and wild-type EGFR.

D. Funding obtained based on work supported by this award

1. DOD 2002 BCRP Idea Award (DAMD 17-02-submitted and award to Dr. Valerie Weaver Title:
Adhesion –linked protein tyrosine phosphatases, morphogenesis and breast cancer progression

E. List of Personnel Receiving Pay

1. Johnathon N. Lakins

Conclusions

21 “classical” protein tyrosine phosphatases (PTPs) were identified in human mammary epithelial cell (MEC) lines. Degenerate RT-PCR of PTP catalytic domains followed by restriction fragment differential display (RFDD) allowed simultaneous assessment of expression levels of a number of these PTPs from the continuous HMT-3522 cell series that includes both non-malignant S1 and tumorigenic T4-2 cells in monolayer and during normal and dysregulated morphogenesis in EHS-ECM (Matrigel). PTP expression is in general higher in tumorigenic T4-2 cells and to be unregulated by Matrigel in which they grow as disorganized masses. In contrast, acinar morphogenesis of non-malignant S1 cells in Matrigel suggests some coordination of regulation of expression in which a number of PTPs are upregulated transiently within the first two days decreasing with varying kinetics by days 4 to 10, at which time the majority of cells are growth arrested. The kinetics of downregulation for some suggests that growth arrest may be the main regulatory input. Others however, downregulate with more rapid kinetics before significant growth arrest suggesting a different regulatory input. Formation of cell-cell adherens junctions may be one such input as ectopic expression a dominant negative E-cadherin construct that blocks cell-cell adherens junction formation delayed but did not prevent downregulation of selected PTPs. Upregulation of actin cytoskeleton regulating PTPs (PTPN21, PTPN4, and PTPN12) may in part be a response of normal MECs to decreases in substrate compliancy (Matrigel vs tissue culture plastic) which result in more cortically organized actin and cell shape changes as normal MCF10A cells express more of these PTPs when cultured on (~1 order of magnitude) softer (tissue-like) Matrigel coupled polyacrylamide gels. PTPN12 is a cytoplasmic actin regulating tyrosine phosphatase that is expressed highly in MECs and is implicated in actin dependent processes such as migration and cytokinesis. Immunofluorescence localization and expression of an EGFP PTPN12 fusion protein showed that PTPN12 was found transiently at the leading edge of lamellopodia of spreading cells and in metaphase cells decorating the mitotic spindle and plasma membrane. Stable downregulation of PTPN12 by retroviral mediated shRNAi resulted derangements of the actin cytoskeleton including the evolution of large filopodial extensions and broad lamellae in MCF10A cells. Such cells had apparently normal rates of division on tissue culture plastic but grew more rapidly and formed larger but yet normally polarized acini in Matrigel. Present studies are aimed at delineating the mechanistic basis for

these morphogenetic effects. It is anticipated that these studies will lead to insights that improve our understanding the details of the structure and regulation of adhesive interactions and PTPs and their relationship to cell fate decisions that will lead to improvements in prognostic, diagnostic and therapeutic approaches to breast cancer.

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